4

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF
PREVENTION, PESTICIDES
AND TOXIC SUBSTANCES

TXR# 0053425

DATE: June 27, 2006

MEMORANDUM

SUBJECT: **MESOTRIONE** – Review of Protocol for Proposed Mouse Developmental Neurotoxicity Study; and Review of Registrant's Submitted Positive and Historical Control Studies

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ACTION REQUESTED: The Registration Division (RD) requested the Health Effects Division (HED) to review a protocol for a proposed developmental neurotoxicity study in mice, as well as positive and negative historical control data for the same. Both were submitted by Syngenta Crop Protection, Inc.

BACKGROUND: At a meeting between HED and Syngenta scientists on June 22, 2005, it was agreed that positive and historical control data would first be submitted to and evaluated by the Agency before a detailed review of the DNT study protocol, submitted under DP# 317658, could be performed. Syngenta's notes for that meeting were submitted under DP# 320021. Syngenta's positive and historical control studies were submitted under DP# 320022. The review of the submitted positive control studies was successfully completed and sent by email directly to Dan Campbell, Senior Regulatory Product Manager at Syngenta Crop Protection, on October 12, 2005. This memo serves as a record of 1) the review of the protocol for the proposed DNT study in mice and 2) the review of the originally submitted positive and historical control data.

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1

CONCLUSIONS

I. Review of Study Protocol for Proposed Developmental Neurotoxicity Study in Mice (MRIDs 46553500-01)

As discussed at the meeting between HED and Syngenta scientists on June 22, 2005, the protocol for the proposed DNT in the mouse is considered unacceptable, until adequate positive and historical control data are submitted to the Agency.

II. Review of Positive and Historical Control Data (MRIDs 46610400-06)

The following review of the registrant's positive and historical control data was emailed to the registrant as a draft document on October 12, 2005. On January 18, 2006, Dan Campbell emailed Syngenta's response to this review to Robert Mitkus and PV Shah of HED and James Stone of RD. This email was followed up with a formal paper submission by Dan Campbell on January 25, 2006, under DP# 326104, to which Robert Mitkus responded on February 21, 2006 (TXR #0053700).

"Points to be discussed for clarification/
Review of Positive Control Studies for Proposed Developmental Neurotoxicity Study Protocol in Mice Treated with Mesotrione (MRID 46553500)

A. Positive Control Studies

A.1 Auditory startle response [CTL/WM0486 (MRID 46610403) and WM0486IAD02_STARTLE.rtf, WM0486TAB01_STARTLEAMP.rtf, and WM0486TAB02_STARTLETIME.rtf]

Adult Alpk:APfCD-1 mice (6-9/sex/group) were treated with clonidine (i.p.) at dose levels of 0, 0.08, or 0.8 mg/kg one hour prior to testing in a San Diego Instruments startle reflex system. Responses (amplitude and time to max. startle) to an auditory stimulus (110 dB) were measured in 5 blocks of 10 trials each. The study is unacceptable for measuring auditory startle response in adults, because habituation was not demonstrated in controls and dose-related decreases in startle amplitude were not demonstrated in females. In addition, dose-related increases in startle amplitude were not measured in the study. The study is unacceptable for measuring developmental auditory startle response, because weanlings were not tested. The following deficiencies contributed to the unacceptability of the study:

- habituation was not tested in weanlings, as proposed in the submitted DNT protocol and required by EPA Guideline OPPTS 870.6300
- information on system calibration was lacking
- the nature of the technical problems with the apparatus were not reported

2

- technical problems with the apparatus decreased the # animals tested at each dose from 10 to 6-9
- habituation was not demonstrated in adult controls
- variability (CV often 100% or more) in overall startle amplitude suggests an inability to detect small differences between treated and control groups,
    - a 32% decrease in startle amplitude in females at 0.08 mg/kg was not detected due to high variability (CV=90%), whereas a 23% overall increase in time to max. amplitude in males at 0.8 mg/kg was detected when variability was low (CV=19%)
    - inter-animal variability was also high, e.g., animal #s 18, 33, 35, 38, 48, 51, 56 did not habituate
- repeated measures analysis was not performed to assess differences between blocks (i.e., habituation) within a given treatment or control group
- the Student's t-test is inappropriate to assess differences between groups when the number of groups > 2; a suitable post-hoc test (e.g., Dunnett's test) is recommended for multiple comparisons against a single control
- measure of data variability (e.g., SD) was not reported for overall startle amplitude or time to max. amplitude for each animal

A.2 Motor activity [CTL/WM0508 (MRID 46610404) and WM0508IAD01_MOTOR.rtf]

Alpk:APfCD-1 mice pups (10/sex/group) were treated (i.p.) with 0 or 1 mg/kg d-amphetamine sulphate or 0 or 5 mg/kg chlorpromazine HCl (CPZ) one hour prior to testing in a San Coulborn Lab Linc Infrared Motion Activity System. Motor activity was measured in 5 blocks of 10 trials each on postnatal days (PNDs) 13, 17, and 21. The study is acceptable for demonstrating increases in overall locomotor activity, relative to controls, on PND 21 only. The study is unacceptable for demonstrating increases in overall locomotor activity, relative to controls, on PNDs 13 and 17. The study is also unacceptable for demonstrating decreases in overall locomotor activity, relative to controls, as well as habituation, on (PNDs) 13, 17, and 21 or in adult mice. The following deficiencies contributed to this decision:

- Motor activity was not tested in adults (PND 60), as proposed in the submitted DNT protocol and required by EPA Guideline OPPTS 870.6300
- information on system calibration was lacking
- animals do not appear to be moving at 5 mg/kg CPZ
    - the following doses of chlorpromazine have been tested in studies published in the open literature:
        - 0.4, 1.2, and 3.6 mg/kg (Simon et al. 2000)
        - 0.2 mg/kg (Messiha 1991)
        - 2 and 5 mg/kg (Nagasaka and Kameyama 1983)
- testing was unable to detect differences in overall motor activity due to CPZ on PND 13

- variability (CV often 100% or more) in overall motor activity suggests inability to detect small differences between treated and control groups
    - testing was able to detect 93% (males) and 78% (females) decreases in overall motor activity due to CPZ on PND 17; however, the ability of the laboratory to detect smaller changes in motor activity was not demonstrated on PND 17
    - inability to detect a 91% decrease in overall motor activity with CPZ in males on PND 13, but ability to detect a 78% change due to CPZ in females on PND 21 was observed
- dose-response curves were not established for either positive control compound
    - "A major purpose of positive control data is to characterize the sensitivity of the test method within the laboratory [43]. Dose–response data are considered very valuable for the successful use of reference compounds during validation [46]. In effect, dose–response data provide a 'calibration' of the test method that documents the ability to detect different magnitudes of effect."[1]
- repeated measures analysis not performed to assess differences between blocks (i.e., habituation) for a given treatment or control group

A.3 Learning and memory

A.3.1 Y-maze [CTL/WM0507 (MRID 46610405)]

Adult Alpk:APfCD-1 mice (20/sex/group) were treated (i.p.) with 0, 0.1, or 0.2 mg/kg (+)-MK 801 maleate 30 minutes prior to measuring the time to escape from a Y-shaped water maze. Time to escape was measured for 6 trials in a Y-maze (dimensions not reported) immediately followed by assessment of time to swim a fixed distance (not reported) in a straight channel on days 1 (acquisition/learning phase) and 4 (memory phase). The study is unacceptable for measuring learning and memory in adults, because the results are confounded by the fact that motor impairment was observed, as evidenced by the increase in straight channel swim time at the high (females) or both (males) doses on day 1. The following deficiencies contributed to the unacceptability of the study:

- Learning and memory were not tested in weanlings (PND 21), as proposed in the submitted DNT protocol and required by EPA Guideline OPPTS 870.6300
- variability in the data was relatively high (CV often >50%)
    - % successful trial data excluded for animals F109, M43, M49, and M54 on day 1, because time to escape and time in straight channel was 30 seconds for most or all trials
- a repeated measures ANOVA should have been performed to compare trials 2-6 with trial 1 (i.e., to assess learning) within control and treated groups
- it is preferable for straight channel swim time to be evaluated before assessment of learning and memory to test for potential motor impairment

[1] Crofton KM, Makris SL, Sette WF, Mendez E, Raffaele KC. (2004). A qualitative retrospective analysis of positive control data in developmental neurotoxicity studies. Neurotoxicol Teratol 26(3), p. 350.

A.3.2 Novel object recognition [CTL/WM0543 (MRID 46610401)]

Pups (25-27 days old) and adult (61-68 days old) Alpk:APfCD-1 mice (10/sex/group) were treated (i.p.) with 0 or 3 mg/kg (-)-scopolamine 1 hour prior to the training session for a novel object recognition task. One hour later, the mice were tested in a retention session. The ratio of time spent at one object relative to a second object in the same open field box (preference index) was calculated for each animal in the training session. A preference index was also calculated for the memory session, in which the time spent at one object relative to a different (novel) object in the same open field box was recorded. The study is unacceptable for measuring learning and memory in pups and adults, due to the following deficiencies:

- a rationale for the appropriateness of novel object recognition (NOR) for associative learning and memory was not provided
- the NOR test does not assess learning as a "change across several repeated learning trials or sessions," as per EPA Guideline OPPTS 870.6300; a learning curve was not established.
- control animals did not retain their object preference after a 3-hr. inter-trial period; this does not support the validity of the test of learning and memory
- no difference in NOR was demonstrated between adults and pups
- only 1 dose of scopolamine was tested
- there appears to have been no control for side preference; it is not known whether the position of the objects, relative to one another, was counterbalanced across treatment groups
- there appears to have been no control for object preference; one object could have simply been more interesting than the other
    - the difference in the size of the objects used in testing could explain the increase in preference index for the novel object in controls
    - the dimensions of each object were not provided
    - habituation or loss of fearfulness, not necessarily learning, might explain the increase in preference index for the second (novel) object in controls
- there appears to have been no control for animal scents; it is not known whether the objects were cleaned after removal from the open field or how often the open field was chamber cleaned
- the distance criterion for judging preference was not clear; was the perimeter marked around the object 2 cm from the center or periphery of each object?
- the time spent exploring either object was relatively short for both control pups and adults (range: 3-66 sec. out of 180 sec.)
- for the preliminary tests with controls, statistical testing was not performed to determine significant differences in preference indices among inter-trial times for either pups or adults

A.4 Brain morphometric measurements and neuropathology with trimethyltin (CTL/RM1004; replaces MRID 46610400, Appendix B)

Adult Alpk:APfCD-1 mice (10/sex/group) were treated (i.p.) with 0 or 2 mg/kg trimethyltin (TMT) on PND 60. On PND 63, a neuropathological evaluation, including brain morphometric and brain weight measurements, was performed on the euthanized animals. The study is unacceptable for measuring TMT-induced changes in brain morphometric measurements and neuropathy in adult mice, because morphometric changes would not be expected in adults after 2 days of treatment with trimethyltin. As importantly, developing mice will need to be tested with a toxicant, e.g., methylazoxymethanol (MAM), at multiple doses and with embedding of all tissues done at the same time. The following deficiencies contributed to the unacceptability of the study:

- While the description of the morphometric measurements provides a reasonable level of detail, it does not provide 1) a description or illustration of the landmarks as they are used at different ages and 2) how the landmarks ensure the consistent identification of a feature. For example, while the description of the measurement of the corpus callosum provides a reliable basis for mediolateral and dorsoventral localization, no specific landmarks were provided for ensuring consistent location in the anterioposterior (AP) dimension. "Block level" is not a sufficient criterion for anterioposterior localization. Instead, specific defining landmarks need to be used for all structures measured.
- photomicrographs with units of measurement, not drawings, are more appropriate for depicting brain regions for morphometry
- scoring criteria and illustrative examples were not included in the micropathology results
- a better marker than the corpus callosum is needed for white matter, specifically the medullary layer
- the caudate-putamen should have been measured due to the known effects of mesotrione on the dopaminergic pathway, i.e., tyrosinemia
- a significant increase in "neuronal degeneration/necrosis/apoptosis" was noted in both sexes, but the specific brain region for this finding was not reported
- morphometry and micropathology not performed on all 10 treated animals, but in $\geq$7 animals
- multiple doses of TMT were not tested
- it wasn't clear why imaging of some sections was performed with a video camera and others with a microscope alone; consistent use of a low power stereoscope/camera or microscope is highly recommended
- brains were weighed and then fixed in this study, whereas in a submitted historical control study [CTL/WM0488 (MRID 46610402)], brains were fixed, then weighed; consistent technique across experiments should be used

B. Historical Control Studies

B.1 Brain morphometric measurements [CTL/WM0488 (MRID 46610402)]

Brain morphometric and measurements were performed on Alpk:APfCD-1 mice (10/sex/group) on PNDs 9, 11, 13, 22, 28, 41, and 62, while brain weights were

measured on PNDs 6, 9, 11, 13, 15, 21, 28, 41, and 62. The study was unacceptable for generating historical control data for any morphometric endpoint, because the laboratory was unable to demonstrate age-dependent differences in almost all brain morphometric parameters in either sex (except for the cerebellum in both sexes). Age-dependent changes in the corpus callosum especially ought to have been detected due to an increase in myelination which takes place from PND 12 to adulthood in rodents. The following deficiencies were very similar to the positive control study with trimethyltin (section A.4 above):

- While the description of the morphometric measurements provides a reasonable level of detail, it does not provide 1) a description or illustration of the landmarks as they are used at different ages and 2) how the landmarks ensure the consistent identification of a feature. For example, while the description of the measurement of the corpus callosum provides a reliable basis for mediolateral and dorsoventral localization, no specific landmarks were provided for ensuring consistent location in the anterioposterior (AP) dimension. "Block level" is not a sufficient criterion for anterioposterior localization. Instead, specific defining landmarks need to be used for all structures measured.
- photomicrographs with units of measurement, not drawings, are more appropriate for depicting brain regions for morphometry
- a better marker than the corpus callosum is needed for white matter, specifically the medullary layer
- the caudate-putamen should have been measured due to the known effects of mesotrione on the dopaminergic pathway, i.e., tyrosinemia
- it wasn't clear why imaging of some sections was performed with a video camera and others with a microscope alone; consistent use of a low power stereoscope/camera or microscope is highly recommended
- brains from PND 6 and 15 animals were not embedded for processing, but were stored for future analysis; this is unacceptable, since all embedding of tissue needs to take place within the same time frame
- brains from PND 6, 9, 11, 13, and 15 animals were weighed and/or processed ≥ 24 hrs. after fixation; however, it was not reported when brains from PND 21, 28, 41, or 62 animals were weighed and processed
- 10 animals/sex were not used for brain morphometric measurements, esp. on PND 9; no reason was provided
- brains were fixed, then weighed, whereas in the TMT study, brains were weighed and then fixed; the use of consistent technique across all experiments and protocols is required

B.2 DNT endpoint measurements (CTL/RM1003; replaces MRID 46610400, Appendix A)

Two groups of Alpk:APfCD-1 dams (≥22/group) were examined for clinical signs, FOB, reproductive performance, body weight, and food consumption from gestation through lactation. The offspring of these dams were examined for body weight, food consumption, and developmental landmarks, as well as for clinical signs and FOB (PND

7

4, 11, 21, 35, 45, 60), motor activity (PND 13, 17, 21, 59), auditory startle (PND 22, 60), novel object recognition (PND 23, 61), brain weight (PND 11, 62), and morphometry (PND 11, 62). The submitted study is unacceptable for generating historical control data for motor activity, auditory startle, learning and memory, and brain morphometry in mouse pups. The validity of the historical control data is dependent on acceptance of validated positive control data for behavioral testing, as well as positive control data for neuropathology, including brain morphometry. The following deficiencies contributed to the unacceptability of the study:

- the test system used to measure motor activity and auditory startle habituation was not reported
- statistical analysis was not performed to assess habituation in motor activity or auditory startle testing
- variability was high (CV=50-100%) for interval and overall data for motor activity on PNDs 21 and 60 (both sexes)
- auditory startle data were quite variable within (CV=50-100% for peak amplitude) and between control groups
- novel object recognition has not been demonstrated to be an appropriate measure of learning and memory, as described in EPA Guideline OPPTS 870.6300
- data from 3 males and females were excluded from auditory startle summary tables on PND 22
- for comments on neuropathology and brain morphometry measurements, refer to sections A.4 and B.1 (above)
- the time, relative to sacrifice, of PND 11 and 62 brain processing was not reported
- the study report states that FOB measurements would be recorded for PND 4, 11, 21, 35, 45, and 60; but they were actually reported for PND 9, 16, 20, and 27 only
- clinical signs and FOB results were not reported for PND 4, 35, 45, and 60
- body weights and food consumption of all maternal animals was not measured on all reported days"